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Immunogenicity of Purified Venezuelan Equine Encephalitis Virus Inactivated by Ionizing Radiation

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Purified and concentrated Venezuelan equine encephalitis (VEE) virus derived from tissue cultures, rendered noninfectious by ionizing radiation with retention of in vitro serological activity, also retained a high level of immunogenicity. In mice, fluid vaccines afforded excellent protection against lethal challenge with homologous Trinidad strain VEE virus. A direct relationship was observed between concentration of vaccine or number of injections and survival. One intraperitoneal inoculation of undiluted vaccine protected essentially all mice challenged 21 days later with 100,000 mouse intraperitoneal LD_{50} of virus. Similarly, mice receiving three injections of vaccines diluted 1:100 were completely protected. Noninfectious VEE virus preparations combined with adjuvant 65, a nontoxic metabolizable vehicle, were likewise very effective in protecting mice immunized intraperitoneally or subcutaneously against lethal challenge. Guinea pigs immunized subcutaneously with adjuvant-combined vaccine survived lethal challenge of 1,000,000 guinea pig intraperitoneal LD_{50}.

Existing vaccines for the protection of laboratory personnel potentially exposed to Venezuelan equine encephalitis (VEE) virus include several Formalin-inactivated preparations (8, 10; Smith et al., Bacteriol Proc., p. 59, 1954.) and a live, attenuated vaccine (2). Unfortunately, the Formalin-treated VEE vaccines either contain residual infectious virus (15) or are so drastically treated as to confer inadequate protection (2, 7). The live, attenuated vaccine, although conferring good protection in man (7), causes a significant incidence of undesirable clinical manifestations (1). An experimental tissue culture vaccine inactivated by ionizing radiation was recently described (13).

In studies to develop a more effective nonviable VEE vaccine, we have investigated the applicability of two approaches not previously utilized in conjunction: purification and concentration of virus by diethylaminoethyl (DEAE) cellulose chromatography and inactivation by ionizing radiation. The procedure proved applicable to obtaining purified and inactivated VEE virus preparations that retained significant in vitro serological reactivity (4). The capability of these serologically active noninfectious virus preparations to confer protection in animals was investigated, and the results are presented in this report.

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MATERIALS AND METHODS

Virus. The Trinidad strain of VEE virus, originally isolated in guinea pigs from a donkey brain (9), was used. It had been passaged 13 times in chicken embryos when it was obtained through the courtesy of our colleague William P. Allen. Working seeds for these studies were prepared from a second suckling mouse brain passage as 10% suspensions of infected suckling mouse brains in 7.5% bovine serum albumin-borate saline.

Infectivity and antigenicity determinations. Assays for virus infectivity were performed by plaque titration in 24-hr chick embryo monolayer tissue cultures (CETC) and by titration of lethality in 10- to 12-g weanling mice. Samples of irradiated virus that failed to produce plaques in tissue culture and were nonlethal for weanling mice after joint intracerebral-intraperitoneal inoculation (0.03 and 0.1 ml) were titrated in suckling mice for residual live virus by a combination intracerebral and intraperitoneal inoculation of 0.02 and 0.03 ml, respectively (12). Fifty per cent lethal dose end points (LD_{50}) were calculated by the method of Reed and Muench (11). Assays for viral hemagglutinins (HA) and complement-fixing (CF) antigens were performed using microtiter methods (3, 14). Goose erthrocytes, at pH 5.8, were used in HA tests, and the Center for Disease Control Laboratory

Branch Complement Fixation (LBCF) procedure was employed in CF tests (16).

Tissue culture. VEE virus was grown in 24-hr chick embryo monolayer tissue culture in a medium consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 10% calf serum, and 0.14% sodium bicarbonate in Hanks balanced salt solution. Infected supernatant fluids were harvested by aspiration at 18 to 20 hr postinfection, and tissue culture cellular debris was removed by slow-speed centrifugation. Prior to chromatography the clarified virus preparations were dialyzed for 18 hr at 5 C against 10 volumes of water to decrease their salt concentration. This procedure did not materially decrease VEE plaque titer or the reactivity of the viral antigen in vitro.

Purification and concentration. VEE virus was purified and concentrated by chromatography of infected CETC supernatant fluids on DEAE cellulose columns (4). After the removal of tissue culture components with 0.01 M NaCl, adsorbed virus was specifically eluted with a 0.7 M tris(hydroxymethyl)aminomethane (Tris)-succinate-salt buffer (TSS), at pH 10. One hundred per cent of the input virus infectivity and viral antigenicity was usually recovered after chromatography. Further concentration and purification was obtained by sedimentation of pooled column eluates on 50% sucrose cushions.

Irradiation. Purified VEE virus vaccines were inactivated by irradiation at the National Bureau of Standards through the courtesy of Daniel W. Brown. Suspensions of virus were exposed to gamma radiation from a cobalt-60 source at a dose rate of 7×10^4 r per min. The virus preparations were irradiated in Wheaton glass serum bottles fitted with flange-type rubber stoppers. Vaccines were kept frozen with dry ice during irradiation to minimize indirect radiation effects and were held in the frozen state until tested for immunogenicity.

Vaccines. Vaccines were prepared from purified VEE virus recovered after chromatographic purification and sucrose cushion sedimentation. Prior to inactivation the virus preparations were diluted 1:4 in either 0.66% L-histidine (free base in borate saline, pH 9.0) or in borate saline alone. Vaccine preparations were irradiated in the frozen state as described above and virus inactivation was verified by tissue culture and animal assay. Details concerning the number of injections, route, volume, and concentration of the individual fluid vaccines are described below. Combination vaccines of virus and adjuvant 65, a mixture of 86% peanut oil, 10% Arlacel A (mannide monooleate), and 4% aluminum monostearate (5), were also prepared. These vaccines were prepared by combining equal volumes of inactivated fluid vaccines with nonirradiated sterile adjuvant 65. Immediately before animal inoculation the frozen fluid vaccines were thawed and emulsions were prepared by repeatedly drawing the mixture of vaccine and adjuvant through a 15-gauge needle.

RESULTS

Inactivation, safety, sterility. Previous reports from this laboratory demonstrated that chromatographically purified VEE virus is inactivated by exposure to 6×10^6 gamma radiation without significant loss of in vitro antigenicity (4). This method and dose of radiation were used to inactivate the vaccines used in this investigation. The inactivation of virus preparations typically obtained is indicated in Table 1. As reported previously, inactivation of infectivity was linear and complete at 6×10^6; in contrast, both HA and CF in vitro antigenicities were usually fully retained.

All the vaccines examined were prepared from chromatographed virus preparations of similar infectivity levels. Table 2 lists the seven vaccines studied, their initial infectious virus content, and the HA and CF antigen activity retained after inactivation. All the vaccines tested were comparable in initial viral infectivity titers and viral antigen content.

The several vaccines were carefully examined for residual live virus after irradiation by tests performed in both tissue culture and animal systems. Usually, at least 10% of a total vaccine

TABLE 1. *Effects of ionizing radiation on infectivity and serological activity of purified VEE virus*

10^6 r	$\log_{10}$ PFU/ml[a]	HA titer[b]	CF titer[c]
0	8.6	128	32
1	6.7	128	32
2	4.4	128	32
3	3.4	128	32
4	1.0	128	32
6	<1.0	128	32

[a] Plaque-forming units in chick embryo monolayer tissue cultures.
[b] Agglutination of goose erythrocytes at pH 5.8; 0.05 ml volume.
[c] Laboratory Branch Complement Fixation procedure; 0.025 ml.

TABLE 2. *Infectivity and in vitro antigenicity of irradiated VEE virus vaccines*

Vaccine	Pre-irradiation infectivity[a]	Postirradiation[b]		
		Infectivity[c]	HA	CF
IFS	9.2	None	512	64
IFH	9.2	None	1024	64
IIFS	8.9	None	512	32
IIFH	9.0	None	512	32
IAS	9.0	None	128	32
IIAH	8.8	None	256	64
IIIAH	9.2	None	1024	128

[a] $\log_{10}$ plaque-forming units ml.
[b] All vaccines exposed to 6×10^6.
[c] Infectivity for tissue culture or lethality for weanling and suckling mice.

volume was used for these safety tests. None of the vaccines produced plaques in tissue culture after inoculation with 10^0 to 10^{-2} diluted material. Both 21-day weanling mice and 1- to 5-day suckling mice survived inoculation with 10^0 to 10^{-2} diluted vaccines. With the later vaccines, the weanling and suckling mice received intracerebral-intraperitoneal injections. None of the surviving mice showed any signs of specific illness, and all animals were observed for at least 14 days. With the last vaccine, guinea pigs were employed as a test animal, and no deaths resulted from inoculation of undiluted irradiated material. No residual live virus was detected by any of the tests employed.

The irradiated vaccines were tested for the presence of bacteria by inoculation of fluid thioglycollate broth and Brain Heart Infusion agar media. Incubation was performed at both 37 C and at room temperature (22 to 25 C). All tests showed the vaccines to be sterile.

Fluid vaccines. Initial studies of the immunogenicity of DEAE cellulose column-purified and concentrated VEE virus were performed with irradiated vaccines without adjuvant. Two fluid vaccines of virus irradiated in borate saline (IFS) or 0.5% histidine (IFH) were tested for their ability to confer protection in mice. Eighteen groups of 8 to 10 (10 to 12 g) male weanling mice were immunized with one, two, or three injections of one of three concentrations of IFS or IFH vaccine. Immunizations were made with 0.2 ml of vaccine by the intraperitoneal route: the multiple inoculations were given at 7-day intervals. Protection assays were performed by intraperitoneal challenge of the injected mice with 5,000 $MIPLD_{50}$ of Trinidad strain VEE virus 21 days after the last immunizing injection. Nonimmunized control mice of the same age and sex were challenged simultaneously, and similar mice were also used to titrate the challenge suspension. The results are presented in Table 3. Both fluid vaccines afforded excellent protection for mice. A direct relationship was observed between concentration of vaccine or number of injections and survival. One immunizing injection of undiluted vaccine (saline or histidine) resulted in survival of all challenged mice. Similarly, three injections of the vaccines diluted as much as 1:100 also afforded considerable protection. Mice receiving one injection of vaccine diluted 1:100 failed to survive challenge. The extent of protection conferred by intermediate dilutions of the vaccines was a function of the number of immunizing injections. At this challenge level the diluted histidine vaccine appeared to confer somewhat better protection than the saline vaccine.

Further immunogenicity studies in mice were conducted with additional saline (IIFS) and histidine (IIFH) fluid vaccines derived from an independent tissue culture harvest, column purification, and inactivation. The groups of mice, the dilutions of vaccines, and the number of immunizing injections used were as noted above. In this study, however, a 0.3-ml volume of vaccine was used for immunization. Protection was assayed by intraperitoneal inoculation of 100,000 $MIPLD_{50}$ of Trinidad strain VEE virus 21 days after the last injection. The results are presented in Table 4. Despite the higher challenge dose used, the high level of protection observed with the previous vaccines was again obtained. Mice receiving three injections of either vaccine diluted 1:100 were completely protected. Mice injected once with undiluted vaccine were similarly protected. A dose response relationship was again

TABLE 3. *Effect of VEE fluid vaccine concentration and number of injections on survival of mice after challenge*[a]

Vaccine	Concn	Survival (%) with		
		One injection	Two injections	Three injections
IFS	10^0	100	100	100
	10^{-1}	40	60	90
	10^{-2}	0	0	22
IFH	10^0	100	100	100
	10^{-1}	70	100	100
	10^{-2}	0	30	100

[a] Intraperitoneal challenge with 5,000 $MIPLD_{50}$ of Trinidad strain VEE virus. None of 10 nonimmunized control mice survived simultaneous challenge.

TABLE 4. *Effect of VEE fluid vaccine concentration and number of injections on survival of mice after challenge*[a]

Vaccine	Concn	Survival (%) with		
		One injection	Two injections	Three injections
IIFS	10^0	100	100	100
	10^{-1}	50	100	100
	10^{-2}	0	70	100
IIFH	10^0	83	100	100
	10^{-1}	70	100	100
	10^{-2}	0	60	100

[a] Intraperitoneal challenge with 100,000 $MIPLD_{50}$ of Trinidad strain VEE virus. None of 10 control mice survived simultaneous challenge.

noted, and the protection afforded by intermediate dilutions of the vaccines was a function of the number of immunizations. In contrast to the previous study, both diluted vaccines appeared to confer similar protection.

Vaccines with adjuvant. The effectiveness of these nonviable purified VEE virus preparations in combination with adjuvant was investigated. In an initial study, seven 10- to 12-g weanling mice were inoculated intraperitoneally with 0.5 ml of an emulsion of equal parts of fluid (saline) vaccine and adjuvant 65 (vaccine IAS). Twenty-one days later they were challenged with 5,000 $MIPLD_{50}$ of Trinidad strain VEE virus. The combination vaccine protected all immunized mice. All nonimmunized mice and control mice receiving adjuvant 65 alone failed to survive simultaneous challenge.

In a more extensive study, two groups of 25 adult (18 to 20 g) mice were immunized via the subcutaneous route with either one or two injections of vaccine combined with adjuvant (IIAH). As before, the combination vaccine consisted of an emulsion of equal parts of fluid (histidine) vaccine and adjuvant 65. The fluid vaccine used in this study was the fourth vaccine derived from an independent tissue culture harvest, column purification, and inactivation. The volume of combined vaccine injected was 0.5 ml, and the multiple injections were 28 days apart. Immunizations were spaced so that both groups of mice were challenged simultaneously. Protection was assayed by intraperitoneal injection of 100,000 $MIPLD_{50}$ of Trinidad strain VEE virus 21 days after the last immunizing injection. Results are presented in Table 5. The combination vaccine afforded the mice excellent protection. All 25 mice that received two immunizations and 24 of the 25 mice that were inoculated only once survived challenge. Furthermore, none of the 49 surviving mice demonstrated any signs of illness. In contrast, all nonimmunized control mice and mice injected with adjuvant 65 alone failed to survive simultaneous challenge.

TABLE 5. *Protection of mice immunized with purified and inactivated VEE virus combined with adjuvant 65*[a]

Inoculum	One vaccine injection		Two vaccine injections	
	Alive/total	Survival (%)	Alive/total	Survival (%)
IIAH vaccine	24/25	96	25/25	100
Adjuvant 65 only	0/8	0	0/8	0
None	0/28	0	—	–

[a] Adult male mice, 18 to 20 g. Intraperitoneal challenge with 100,000 $MIPLD_{50}$ of Trinidad strain VEE virus.

TABLE 6. *Protection of guinea pigs immunized with purified and inactivated VEE virus combined with adjuvant 65*[a]

Inoculum	One vaccine injection		Two vaccine injections	
	Alive/total	Survival (%)	Alive/total	Survival (%)
IIAH vaccine	15/15	100	15/15	100
Adjuvant 65 only	0/2	0	—	
None	0/15	0	–	

[a] Adult guinea pigs, 250 to 300 g. Intraperitoneal challenge with 1,000,000 $GPIPLD_{50}$ of Trinidad strain VEE virus.

Immunization of guinea pigs. To determine further the protection conferred by these adjuvant-combined nonviable VEE vaccines, immunogenicity studies were extended to include the guinea pig. This species was selected because it made available for further serological and pathological studies a 10-fold larger animal and also because of the guinea pig's marked sensitivity to lethal VEE virus infection. Two groups of 15 young adult guinea pigs (250 to 300 g) were immunized by one or two subcutaneous inoculations of 0.5 ml of a new adjuvant-combined vaccine (IIIAH). The fluid-virus portion of this vaccine represented the fifth independent growth, purification, and inactivation of VEE virus. Multiple injections were spaced 28 days apart, and immunizations were scheduled so that both groups of guinea pigs were challenged at the same time. Immunogenicity was determined by protection from a lethal intraperitoneal challenge of 1,000,000 $GPIPLD_{50}$ of Trinidad strain VEE virus 21 days after the last immunizing injection. Results are given in Table 6. The combination vaccine completely protected the guinea pigs. Despite the massive challenge employed, all 30 immunized animals survived, regardless of whether they received one or two immunizing injections. None of these animals showed any indication of illness throughout the postchallenge observation period. In contrast, nonimmunized control guinea pigs and guinea pigs receiving adjuvant 65 alone showed signs of nervous system involvement 3 to 5 days after simultaneous challenge, and all died early in the observation period.

DISCUSSION

The development of highly immunogenic nonreactogenic virus vaccines with increased specific

antigen concentration and minimal nonimmunogenic constituents is a common aim of those studying new approaches to viral immunization. Earlier reports from this laboratory indicated that VEE virus preparations of high purity and concentration were obtained by DEAE cellulose chromatography and that ionizing radiation destroyed the infectivity of such preparations without significant damage to their in vitro (HA, CF) antigenic activity. This present report indicates that these purified and inactivated preparations are capable of stimulating a marked response in host animals, resulting in in vivo protection against lethal challenge, and that they are thereby of high immunogenic potency.

Inactivated vaccines of high potency prepared from viruses with unusual stability and resistance to inactivation require exacting safety tests to exclude the potential presence of residual live virus. In this study, three different criteria were routinely used to measure virus inactivation: lack of plaque formation in CETC, lack of lethality for weanling mice, and lack of lethality for very susceptible newborn mice. Additional evidence of inactivation is available from the vaccine studies themselves. The failure of immunized weanling mice to die during the holding period after an initial immunizing injection, where a substantial volume of undiluted and diluted vaccine was employed, provides an even more sensitive indication of the absence of residual live virus in the irradiated preparations.

Further evidence of virus inactivation and an additional indication that immunogenic stimulation by the vaccines is not due to subclinical infection by trace amounts of residual live virus are apparent in the results of the fluid vaccine studies in mice. Excellent protection of immunized animals was obtained with vaccines diluted as much as 1:100 after inactivation and safety testing of the preparations. To attribute the protection observed to potential residual live virus would require an assumption that the assay systems are not capable of detecting live virus in excess of 2 $\log_{10}$. This is obviously not the case, and the immunogenicity observed is evidently a property of the inactivated virus.

The immunogenic potency of the nonviable vaccines described in this report is similar to that of other recently described VEE vaccines that were also inactivated by ionizing radiation (13). Those vaccines were evaluated by determination of the 50% effective dose (ED_{50}), the quantity of undiluted vaccine protecting 50% of tested animals from a lethal challenge. The most potent tissue culture vaccine of several described for mice (ED_{50} of 0.0036) apparently required two immunizing inoculations of a 1:70 dilution of vaccine to protect half of the mice. Two vaccines that were partially purified by ultracentrifugation apparently required two (ED_{50} of 0.01) and three (ED_{50} of 0.017) immunizations of 1:25 dilution of virus for best effect. Our studies of diluted fluid vaccines in mice furnish similar data from which valid comparisons can be made. Mice inoculated twice with purified VEE vaccines (IIFS, IIFH) and subjected to an equivalent lethal challenge (Table 4) were completely protected with 1:10 diluted vaccine and exhibited more than 50% survival with vaccines diluted 1:100 (this represents an ED_{50} of <0.0030). Additionally, these purified vaccines provided 50% protection to mice at 1:10 dilution with only one inoculation and 100% protection to all mice when three immunizations of 1:100 diluted vaccines were used. With guinea pigs, direct comparisons are not possible since we used an adjuvant-combined vaccine administered via the subcutaneous route, but it should be noted that the purified and adjuvant-combined vaccine protected 100% of guinea pigs receiving only a single immunization.

The susceptibility of mice to peripheral inoculation with most arboviruses, in contrast to intracerebral inoculation, usually diminishes markedly with increasing age. This decreasing susceptibility presents problems in vaccine studies where mice are held to maturity before being challenged. However, intraperitoneal neutralization tests with VEE virus were shown to give uniform results in mice of any age up to at least 200 days, and the quantity of virus neutralized was 1,200 to 20,000 times that neutralized by the intracerebral route (6). Thus, survival of adult mice after lethal intraperitoneal challenge is a valid test for evaluating immunity to VEE virus.

Adjuvant 65 is a nontoxic and metabolizable oil vehicle for aqueous vaccines. It has been most thoroughly tested in its application to influenza virus vaccines and has proved highly effective in potentiating the level and duration of antibody both in animals and in man (5). It seemed desirable to determine the applicability of this adjuvant to the VEE virus vaccines under investigation. The VEE virus-adjuvant-combined vaccines in mice proved to be at least as effective as similar fluid vaccines. Weanling mice inoculated intraperitoneally and adult mice inoculated subcutaneously were both afforded essentially complete protection with one inoculation of combined vaccine. Guinea pigs inoculated once and subjected to an extremely high challenge dose were similarly protected. No incompatibility with VEE virus immunogens was noted, and no macroscopic lesions or other pathology was observed at the inoculation sites. Further studies of the immunological protection conferred by adjuvant

65 combined VEE virus vaccines and comparisons with other types of vaccines appear warranted.

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